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# Lead-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation

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## ABSTRACT

Formation of organometallic complexes in soil solution strongly influence metals phytoavailability. However, only few studies deal with the influence of metal speciation both on plant uptake and genotoxicity. In the present study, *Vicia faba* seedlings were exposed for 6 h in controlled hydroponic conditions to 5  $\mu$ M of lead nitrate alone and chelated to varying degrees by different organic ligands. Ethylenediaminetetraacetic acid and citric acid were, respectively, chosen as models of humic substances and low weight organic acids present in natural soil solutions. Visual Minteq software was used to estimate free lead cations concentration and ultimately to design the experimental layout. For all experimental conditions, both micronucleus test and measure of lead uptake by plants were finally performed. Chelation of Pb by EDTA, a strong chelator, dose-dependently increased the uptake in *V. faba* roots while its genotoxicity was significantly reduced, suggesting a protective role of EDTA. A weak correlation was observed between total lead concentration absorbed by roots and genotoxicity ( $r^2=0.65$ ). In contrast, a strong relationship ( $r^2=0.93$ ) exists between  $Pb^{2+}$  concentration in exposure media and genotoxicity in the experiment performed with EDTA. Citric acid induced labile organometallic complexes did not demonstrate any significant changes in lead genotoxicity or uptake. These results demonstrate that metal speciation knowledge could improve the interpretation of *V. faba* genotoxicity test performed to test soil quality.

## 1. Introduction

Lead is one of the most useful and toxic metals present in the environment on a global scale (Sharma and Dubey, 2005; Arshad et al., 2008; Uzu et al., 2009). When exposed to this metal, even at low concentrations, plants usually experience harmful effects such as micronucleus induction (National Toxicology Program, 2003), mitosis disturbance (Wierzbicka, 1999), DNA damage (Gichner et al., 2008), alterations in membrane permeability (Sharma and Dubey, 2005) and disturbance (inhibition or activation) of enzymatic activities (Reddy et al., 2005) along with various physiological impacts.

Recent literature indicates that the toxicity and/or bioavailability of trace metals, in addition to their mobility, shows marked dependence on their speciation (Uzu et al., 2009) and these responses often correlate best with the activity of free metal ion (Dumat et al., 2001; Doig and Liber, 2007). Interactions between organic compounds and metals in natural media have been particularly

studied due to their strong effects on metal behaviour (Quenea et al., 2009). These organic compounds, when present in growth medium, can adsorb or complex metals, affecting their mobility, uptake and even cytotoxicity. Moreover, it was observed that lead can be accumulated and bound within the polysaccharides of cell walls (Sharma and Dubey, 2005). Therefore, in addition to knowing the total metal concentration, predicting the relevant species of metals is of utmost importance in improving our understanding of the mechanisms of metal uptake, accumulation and cytotoxicity.

The use of short-term bioassays, especially genetic toxicity bioassays, to assess potent environmental pollutants has gained special attention over the last decades. These assays are capable of predicting the genotoxic potential of the pollutant under investigation by measuring gene mutations and damage to chromosomes and DNA. Plant assays are quite easy to conduct, inexpensive, rapid and good predictors of genotoxicity (Panda and Panda, 2002). In particular, the *V. faba* micronucleus test is a very sensitive and useful method for the detection of both clastogenic and aneugenic effects (Duan et al., 2000; El Hajjoui et al., 2007; Marcato-Romain et al., 2009).

Lead-induced genotoxicity to *V. faba* was studied using current contact with aqueous extract or direct contact with soil by Marcato-Romain et al. (2009). Due to lower genotoxicity observed

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in the case of direct contact with soil, these authors concluded to a potential influence of soil organic matters. As soil solution represents the main available compartment for metals uptake by plants (Degryse et al., 2009; Gandois et al., 2010), one scientific question asked is the relation between aqueous extract of soil used in current genotoxicity to *V. faba* test and soil solution. Therefore, the objectives of the present study were to identify the correlation between Pb speciation, phytoavailability and genotoxicity to *V. faba* as a function of organic chelates nature and levels.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Dry seeds of broad beans (*V. faba* L. stored at 4 °C) were germinated on moistened filter paper in a germination chamber under optimal conditions of germination, i.e. in darkness at 22 °C temperature and 100% humidity. After 5–7 days, when the primary roots were about 2–3 cm in length, the seedlings were transplanted to a PVC tank (3 plants per tank) containing continuously aerated modified Hoagland nutrient solution (see Uzu et al., 2009 for more detail) with the macro-elements: 5 mM KNO<sub>3</sub>, 5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub> and 1.5 mM MgSO<sub>4</sub> and micro-elements: 9.11 mM MnSO<sub>4</sub>, 1.53 mM ZnSO<sub>4</sub>, 0.235 mM CuSO<sub>4</sub>, 24.05 mM H<sub>3</sub>BO<sub>3</sub>, 0.1 mM Na<sub>2</sub>MoO<sub>4</sub> and 268.6 mM Fe/EDTA. Nutrient solution was renewed on alternate days to keep its composition and pH constant.

After pre-culturing for 15 days, plants were exposed for 6 h to 5 µM lead alone or chelated by EDTA or citric acid (Table 1). EDTA, a strong organic chelator for metals, was chosen to act as a model compound for humic substances (Lai and Chen, 2005; Meers et al., 2005) and citric acid was chosen to model low-molecular-weight organic acids (Chen et al., 2003; Muhammad et al., 2009). The concentration of 5 µM Pb(NO<sub>3</sub>)<sub>2</sub> was chosen because according to Pourrut et al. (2008), it induces genotoxicity for *V. faba* while remaining quite low and representative of the pollution of the environment. The concentration of KH<sub>2</sub>PO<sub>4</sub> in the nutrient solution used for Pb treatment was reduced to 0.2 mM in order to prevent phosphate precipitation (Kopittke et al., 2008; Waranusantigul et al., 2008). Control plants were also cultured in the appropriate uncontaminated media with reduced PO<sub>4</sub><sup>3-</sup> concentration. All plants were grown under controlled conditions in a 16 h photoperiod at 70% relative humidity and day/night temperatures of 24/22 °C. Light was supplied by 600 W Osram Nav-T Super High Pressure Sodium Lamps providing a minimum photosynthetic photon flux density of 500 µmol m<sup>-2</sup> s<sup>-1</sup> at the top of the plant (Pourrut et al., 2008).

### 2.2. Determination of lead concentration and speciation in solution with Visual Minteq

A 50 µM lead solution was first prepared from Pb(NO<sub>3</sub>)<sub>2</sub> and mQ water. That concentrated solution was then filtered (0.22 µm), acidified to pH 5.0 with distilled

HNO<sub>3</sub> (15 M, suprapur 99.9%) and stored at 4 °C before lead analysis by inductively coupled plasma-atomic emission spectrometry (ICP-AES) with an IRIS Intrepid II XDL. The measured lead concentration was 50.02 µM. That concentrated lead solution was then diluted (1/10) in mixture with mQ water or ethylenediaminetetraacetic acid (EDTA) or citric acid (CA) in order to obtain the various exposure solutions (lead alone or with organic ligands). The pH was adjusted to 5 with distilled HNO<sub>3</sub> (15 M, suprapur 99.9%) and lead concentration was checked on ten replicates: 5.01 ± 0.02 µM Pb. Without contact with plants, both pH and total lead concentration measured for the various exposure solutions, in function of time, stayed constant after 6 h. Therefore, for the various estimations of lead chemical speciation with Visual Minteq software a total lead concentration of 5 µM was used.

The Visual Minteq software version 2.60 (Gustafsson, 2008) was used to calculate the concentration of EDTA and citric acid required for the chelation of 15, 25, 40, 75 and 99% of the Pb contained in the nutrient solution. Visual Minteq is a chemical equilibrium model extensively used in the literature (Ge et al., 2005; Doig and Liber, 2007) for the accurate calculation of metal speciation, precipitation and dissolution. Metal ion speciation is calculated using equilibrium constants from the Minteq database (Table 2). The concentration of the elements present in modified Hoagland solution, 5 µM Pb and EDTA or citric acid at different concentrations was used as the input for the Visual Minteq model, at pH 5.0, 25 °C and an ionic strength of 0.1 M (Table 1). The Visual Minteq speciation showed that 1.45, 2.25, 4.25 and 10 µM EDTA are required for 25, 40, 75 and 99% chelation of 5 µM Pb, respectively, while 300, 550 and 1000 µM CA are required for 15, 25, and 40% Pb chelation, respectively, under the same experimental conditions (Table 1). These calculations were used for fitting the experimental design (Table 1). In the experiments, the toxic effect of higher concentrations of citric acid alone (> 1000 µM) restricted the Pb chelation up to 40% and limited its comparison with Pb-EDTA-75 and Pb-EDTA-99.

**Table 2**

Equilibrium constants used for the prediction of Pb speciation in nutrient solution. Equilibrium constants are the default values of the Minteq database from Visual Minteq 2.60 (Gustafsson, 2008).

Reactions	Log K
Pb <sup>2+</sup> + H <sub>2</sub> O ⇌ PbOH <sup>+</sup> + H <sup>+</sup>	− 7.6
Pb <sup>2+</sup> + 2H <sub>2</sub> O ⇌ Pb(OH) <sub>2</sub> + 2H <sup>+</sup>	− 17.09
Pb <sup>2+</sup> + 3H <sub>2</sub> O ⇌ Pb(OH) <sub>3</sub> <sup>−</sup> + 3H <sup>+</sup>	− 28.09
Pb <sup>2+</sup> + Cl <sup>−</sup> ⇌ PbCl <sup>+</sup>	1.55
Pb <sup>2+</sup> + NO <sub>3</sub> <sup>−</sup> ⇌ PbNO <sub>3</sub> <sup>+</sup>	1.17
Pb <sup>2+</sup> + SO <sub>4</sub> <sup>2−</sup> ⇌ PbSO <sub>4</sub>	2.69
Pb <sup>2+</sup> + 2SO <sub>4</sub> <sup>2−</sup> ⇌ Pb(SO <sub>4</sub> ) <sub>2</sub> <sup>2−</sup>	3.47
Pb <sup>2+</sup> + PO <sub>4</sub> <sup>3−</sup> + 2H <sup>+</sup> ⇌ PbH <sub>2</sub> PO <sub>4</sub> <sup>+</sup>	21.07
Pb <sup>2+</sup> + Citrate <sup>3−</sup> ⇌ Pb–Citrate <sup>−</sup>	5.67
Pb <sup>2+</sup> + Citrate <sup>3−</sup> + H <sup>+</sup> ⇌ PbH–Citrate	10.29
Pb <sup>2+</sup> + EDTA <sup>4−</sup> ⇌ PbEDTA <sup>2−</sup>	19.71

**Table 1**

Experimental design and speciation of Pb in modified Hoagland solution under EDTA and citric acid calculated using Visual Minteq 2.60 (Gustafsson, 2008). In treatments notation, the numbers 15, 25, 40, 75 and 99 represent % of Pb chelated by EDTA and citric acid. Lead concentration was kept constant at 5 µM in all treatments except controls. The treatments without Pb (PC, EDTA-75, EDTA-99, CA-25, CA-40) were only used in the micronucleus test as controls.

Treatments	Visual Minteq input values	Visual Minteq calculations	
Notations	Treatments medium composition	Pb-chelated (%)	Pb-free (%)
Pb	HS <sup>a</sup> +Pb(NO <sub>3</sub> ) <sub>2</sub> (5 µM)	0	85
Pb-EDTA-25	HS+Pb(NO <sub>3</sub> ) <sub>2</sub> +EDTA (1.45 µM)	25	64
Pb-EDTA-40	HS+Pb(NO <sub>3</sub> ) <sub>2</sub> +EDTA (2.25 µM)	40	51
Pb-EDTA-75	HS+Pb(NO <sub>3</sub> ) <sub>2</sub> +EDTA (4.25 µM)	75	21
Pb-EDTA-99	HS+Pb(NO <sub>3</sub> ) <sub>2</sub> +EDTA (10 µM)	99	1
Pb-CA-15	HS+Pb(NO <sub>3</sub> ) <sub>2</sub> +citric acid (300 µM)	15	73
Pb-CA-25	HS+Pb(NO <sub>3</sub> ) <sub>2</sub> +citric acid (550 µM)	25	64
Pb-CA-40	HS+Pb(NO <sub>3</sub> ) <sub>2</sub> +citric acid (1000 µM)	40	51
<b>Controls without Pb</b>			
NC <sup>b</sup>	Hoagland solution (HS)	–	–
PC <sup>c</sup>	HS+Maleic hydrazeide (40 µM)	–	–
EDTA-75	HS+EDTA (4.25 µM)	–	–
EDTA-99	HS+EDTA (10 µM)	–	–
CA-25	HS+citric acid (550 µM)	–	–
CA-40	HS+citric acid (1000 µM)	–	–

<sup>a</sup> Hoagland solution (concentrations in introduction).

<sup>b</sup> Negative control.

<sup>c</sup> Positive control.

### 2.3. Lead content analysis

Cell lead was assayed as described by Pourrut et al. (2008). After 6 h exposure, *V. faba* seedlings were harvested, roots were rapidly washed in distilled water and the lead bound to the rhizoderm was removed by 0.01 M HCl according to Ferrand et al. (2006). The roots were washed by shaking for another 5 min in distilled water. After harvest, each plant sample was dried at 50 °C for 48 h before digestion in a 1:1 mixture of HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> at 80 °C for 4 h and then in a hot aqua regia. After filtration, lead concentration was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) with an IRIS Intrepid II XDL. The accuracy of the acidic digestion and analytical procedures was checked using the reference material Virginia tobacco leaves, CTA-VTL-2, ICHTJ. Certified values for lead in tobacco leaves were given for 22.1 ± 1.2 mg Pb kg<sup>-1</sup> dry weights. Measured values for the three replicates were 22.0 ± 0.9, 22.4 ± 0.8 and 21.9 ± 0.8 mgPb kg<sup>-1</sup> dry weight.

### 2.4. *V. faba* micronucleus test

The micronucleus test was carried out according to Ma et al. (1995) and El Hajjouji et al. (2007). Before the test, the primary root tip was cut off (2 mm) to stimulate the emergence of secondary roots before transplanting into hydroponic conditions. Four days were necessary to obtain secondary roots of suitable length (1–2 cm) for the test. Exposure time was 30 h (6 h for the treated groups followed by a 24 h recovery period). In view of lead toxicity after 24 h of exposure (blackening of the root tips and loss of mitosis), a 24 h recovery period was enough to develop micronuclei (AFNOR, 2004; El Hajjouji et al., 2007; Marcato-Romain et al., 2009). Maleic hydrazide (MH, Sigma Chemical Co., St. Louis, MO, USA) at the concentration of 40 µM was used as the positive control and aerated Hoagland's solution alone was used as the negative control (Table 1). For each treatment and controls, five plants were used.

After harvest, root tips were fixed in Conroy's solution (glacial acetic acid/ethanol 1:3 v/v) at 4 °C for 24 h and kept in 70% alcohol at 4 °C. Subsequently, the roots were washed with distilled water for 10 min and hydrolysed with 1 M HCl at 60 °C for 6–7 min. For each replicate, five slides were prepared. Finally, the root tips were stained with an aqueous solution containing 1% aceto-orcein. The slides were observed under 1000 × magnification using an Olympus BX41 microscope coupled with a camera imaging FA1394-Q RETIGA Exi. Lead-induced genotoxicity was recorded as permillage (number of micronuclei per 1000 cells) micronuclei (MN) frequency. For each root tip, 5000 cells were observed. In order to avoid underestimation of micronucleus frequency due to impaired cell proliferation rate, the micronucleus test was performed only on root tips with a mitotic index of over 2% (AFNOR, 2004).

### 2.5. Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) using the software Statistica, ver. 8 (StatSoft-France). A Tukey's honestly significant difference (HSD) test was used to determine the level of significance against the negative control or Pb alone values.

## 3. Results

### 3.1. Pb uptake by *V. faba* roots in the presence of organic ligands

The uptake of lead by *V. faba* roots in the presence of EDTA and citric acid is depicted in Table 3. The Pb concentrations in roots exposed to Pb alone reached an average value of 61 ± 5.9 mg kg<sup>-1</sup> DW after 6 h incubation. Addition of EDTA led to a significant increase in root Pb concentration (18, 25, 34 and 33% increase for Pb-EDTA-25, Pb-EDTA-40, Pb-EDTA-75 and Pb-EDTA-99, respectively) as compared to Pb alone. The effect of EDTA on Pb uptake by *V. faba* roots was concentration-dependent, except for Pb-EDTA-99. In this experimental condition, the Pb concentration in Pb-EDTA-99 roots was no longer different than Pb-EDTA-75. With citric acid, only a slight but non-significant increase of Pb uptake in *V. faba* roots was observed in comparison with uptake of Pb alone (3, 9 and 7% for Pb-CA-15, Pb-CA-25 and Pb-CA-40, respectively).

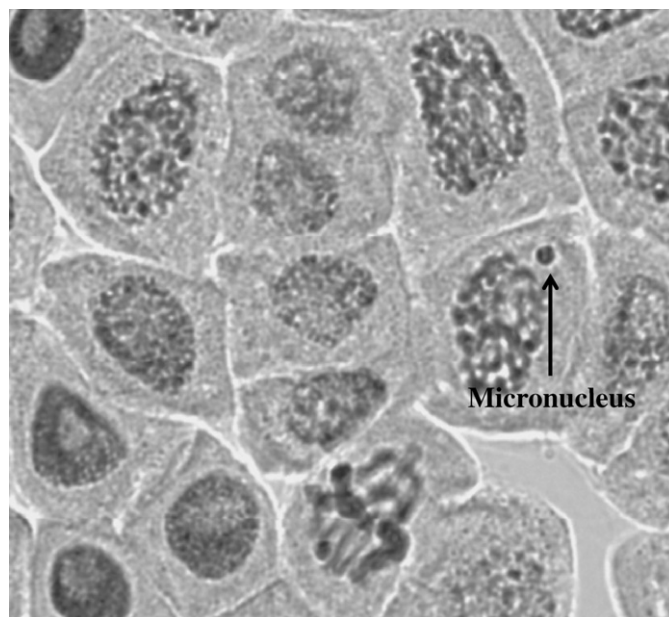
### 3.2. Lead-induced genotoxicity in the presence of organic ligands

When EDTA or citric acid alone (as control) was added to the nutrient solution, no significant effect was observed on

**Table 3**

Effect of EDTA and citric acid on Pb availability in nutrient solution (calculated using Visual Minteq software) and Pb accumulation in whole plant *V. faba* roots. Values of Pb accumulation are means of three separate experiments each replicated five times. Different letters among treatments indicate significant differences at  $P < 0.05$ .

Treatment	Pb available (% increase)	Pb in roots (mg kg <sup>-1</sup> )
Pb	–	61 ± 5.9 a
Pb-EDTA-25	5	73 ± 6.0 abc
Pb-EDTA-40	7	77 ± 4.3 bc
Pb-EDTA-75	13	82 ± 5.6 c
Pb-EDTA-99	18	81 ± 4.9 c
Pb-CA-15	4	63 ± 5.1 ab
Pb-CA-25	5	67 ± 3.9 ab
Pb-CA-40	7	66 ± 5.2 ab

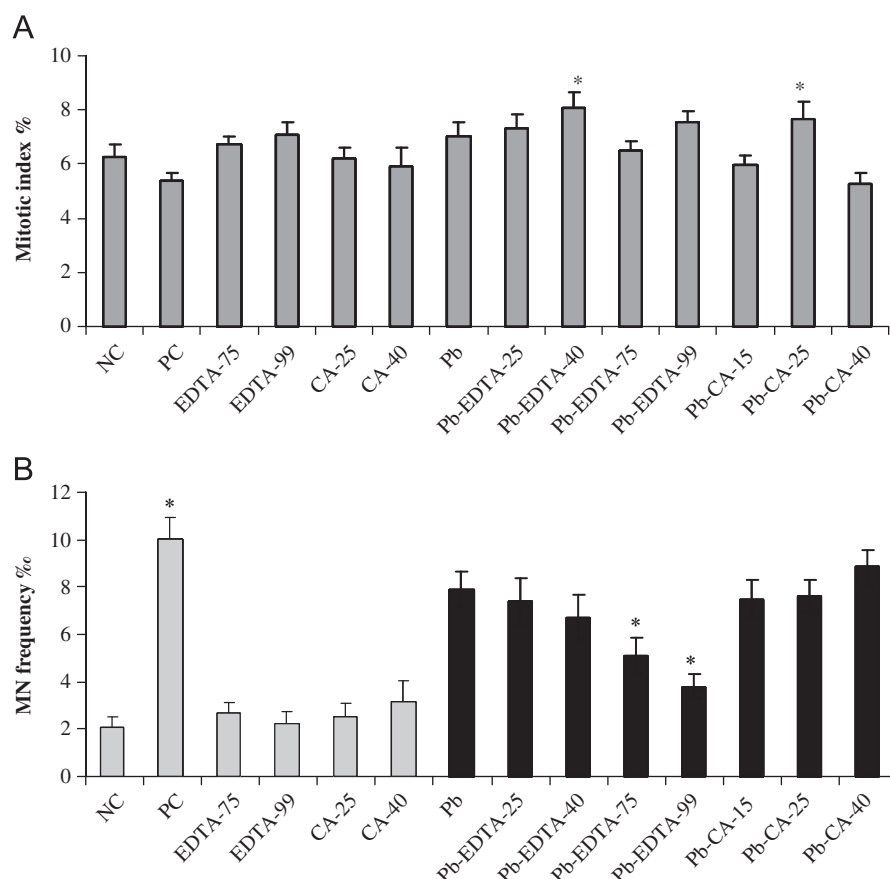


**Fig. 1.** Micronucleus induced by lead in *V. faba* root cells. Micronucleus is marked with an arrow.

micronucleus frequency or mitotic index compared to the negative control (Fig. 2A and B). However, a toxic effect was observed for high concentrations of citric acid (> 1 mM; results not shown) and limited its use to 40% chelation of Pb (Pb-CA-40). Türkoğlu (2007) also reported a significant decrease in mitotic index with citric acid alone in *Allium cepa* L.

When *V. faba* seedlings were exposed to Pb alone, the micronucleus frequency increased significantly. A digital picture of the micronucleus induced by lead in *V. faba* root tips is presented in Fig. 1. A four-fold increase in micronucleus frequency compared to the negative control was observed for Pb alone (Fig. 2B). Addition of EDTA did not affect the mitotic index, except Pb-EDTA-40, which caused a significant increase compared to Pb alone. However, in the presence of EDTA, lead-induced micronucleus frequency (indicative of genotoxicity) decreased significantly and dose dependently. Chelation of 25, 40, 75, and 99% of the Pb by EDTA reduced lead-induced micronucleus frequency by 6, 15, 35 and 52%, respectively.

Application of citric acid decreased mitotic index at the concentrations Pb-CA-15 and Pb-CA-40 but the effect was non-significant (Fig. 2A). Addition of citric acid, however, had no significant effect on Pb genotoxicity (Fig. 2B). In these



**Fig. 2.** Mitotic index (A) and micronucleus frequency (B) values in *V. faba* root tips exposed to Pb alone and Pb chelated with EDTA and citric acid. In Fig. 2B, the black and grey bars represent treatments with and without Pb, respectively, in order to compare their MN frequencies separately. Values are means of three separate experiments each replicated five times. The symbol (\*) among treatments indicates significant differences at  $P < 0.05$ . (NC, negative control; PC, positive control).

experimental conditions, the micronucleus frequency induced by lead in the presence of citric acid remained close to that with Pb alone, except for Pb-CA-40, which increased slightly (16%).

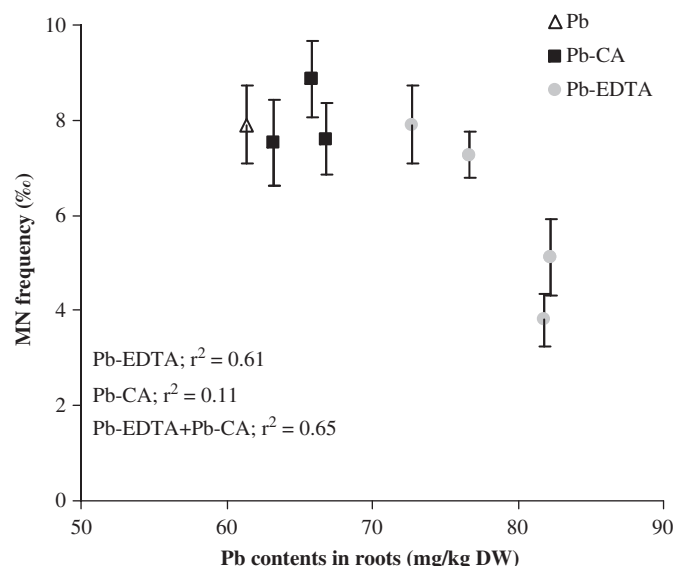
### 3.3. Relationship between genotoxicity and Pb concentrations

No correlation was found between genotoxicity and total Pb concentration in *V. faba* roots tips under both EDTA and citric acid ( $r^2=0.65$ ) as shown in Fig. 3. The results were similar when correlation was calculated separately for EDTA ( $r^2=0.61$ ) and citric acid ( $r^2=0.06$ ) treatments (data not shown). However, a linear curve was obtained for EDTA ( $r^2=0.93$ ) when genotoxicity was plotted against the  $Pb^{2+}$  concentration in solution (Fig. 4). Such a relation ( $r^2=0.41$ ) was not found between micronucleus frequency and  $Pb^{2+}$  concentration in solution for citric acid (data not shown).

## 4. Discussion

### 4.1. Lead uptake by *V. faba* roots in the presence of organic ligands

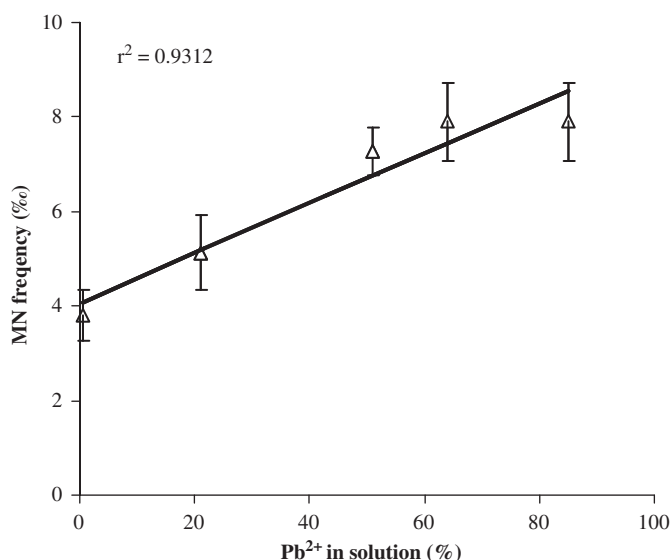
As expected, the addition of EDTA significantly increased Pb uptake by *V. faba* roots (Table 3). This result is in agreement with several previous studies (Lai and Chen, 2005; Luo et al., 2005; Meers et al., 2005), which reported many fold increase in Pb uptake by various plants in the presence of EDTA. Chen et al. (2004) observed that the addition of EDTA (2.5 and 5 mmol  $kg^{-1}$  EDTA) to a Pb-contaminated soil (2400 mg  $kg^{-1}$  of total soil Pb) increased shoot Pb concentrations of ten plant species by 24–104



**Fig. 3.** Micronucleus (MN) frequency plotted against total Pb concentration in *V. faba* roots in the presence of EDTA and citric acid. Values are means of three separate experiments each replicated five times.

fold, with the greatest increases in dicotyledonous species. It has been demonstrated that Pb uptake is correlated with the formation of Pb-EDTA in the hydroponics solution. Due to its high complexation constant value ( $\log K=17.88$ ), Pb-EDTA is the principal form of Pb to be taken up and translocated in the plants





**Fig. 4.** Micronucleus (MN) frequency plotted against free  $Pb^{2+}$  ion concentration in solution in the presence of EDTA. Values are means of three separate experiments each replicated five times.

(Ruley et al., 2006). However, data presented in Table 3 indicated that no difference in lead uptake was observed between Pb-EDTA-75 and Pb-EDTA-99, suggesting a possible saturation of Pb accumulation after 6 h of incubation.

In the Pb-CA treatments, however, Pb accumulation in *V. faba* roots was slightly higher than with Pb alone, but no treatment reached a significantly different concentration after 6 h of incubation (Table 3). Similar results were observed by Quartacci et al. (2006) who reported that citric acid applied at  $5 \text{ mmol kg}^{-1}$  to a metal-contaminated soil did not induce any significant change in metal uptake by *Brassica juncea*. However, some authors (Chen et al., 2003; Muhammad et al., 2009) also indicated increased absorption of Pb by application of citric acid. The increase in metal uptake by citric acid is plant-dependent and could be attributed to a decrease in the pH of culture media. Acidification did not occur in our experimental condition, because the pH was kept adjusted at 5. Moreover, the slight decrease in Pb uptake by Pb-CA-40 than Pb-CA-25 might be due to the acute cytotoxicity associated with CA. Infact, at higher concentrations CA alone reduced mitotic index and increased genotoxicity, the effects being non-significant (Fig. 2A and B).

#### 4.2. Micronucleus test and mechanism of lead-induced genotoxicity

Four-fold increases in micronucleus frequency in *V. faba* root tips by Pb alone (Fig. 2B) clearly illustrated Pb genotoxicity, analysed with the *V. faba* micronucleus test. The molecular mechanism of DNA breakdown under metal stress is not yet clearly understood. Micronuclei are the result of chromosome breaks or mitotic anomalies that require a passage through mitosis to be recognisable (Al Sabti and Metcalfe, 1995). According to Johnson (1998) lead and its compounds are capable of interfering with the spindle apparatus of dividing cells thus leading to genotoxicity. Bonacker et al. (2005) thought that the micronuclei induced by lead ions are most often associated with processes involving reactive oxygen generated by redox shuttling. Oxidative stress as a result of metal toxicity is also described to play a major role in DNA-damage induction (Halliwell, 1990). In this experiment, a blackening of root tips grown under lead nitrate alone or Pb-CA complexes was observed. Recently Probst

et al. (2009) also found dark coloured *V. faba* roots grown on mine tailings contaminated with Pb. This root coloration is possibly due to lead-induced oxidative stress (Pourrut et al., 2008).

#### 4.3. Relationship between lead uptake and genotoxicity in the presence of organic ligands

Cytotoxicity and bioavailability/uptake are generally considered synonymously when one speaks of metal species. In our experimental conditions, a non-linear correlation was observed between total lead concentration in *V. faba* roots and genotoxicity (Fig. 3) under applied organic ligands. Application of Pb-EDTA treatments to *V. faba* seedlings significantly increased Pb uptake but concomitantly reduced Pb genotoxicity (Table 3; Fig. 2B). These results are in line with those of Liu et al. (2008) in *Sedum alfredii* or Ruley et al. (2006) who studied *Sesbania drummondii*, a lead hyper-accumulator. Hernández-Allica et al. (2007) also reported that proper management of the EDTA concentration can increase the uptake of metals with low phytoavailability like Pb and reduce their phytotoxicity. Recently, Marcato-Romain et al. (2009) presented that MN frequencies in *V. faba* root tips were more than ten-fold higher in hydroponic exposure compared to direct contact in soil under the same range of pollutant concentrations. These observations could be related to lead speciation (different between soil and solution), with a particular protecting effect of soil organic matter. However, citric acid treatments increased, although not significantly, lead-induced genotoxicity with increase in lead uptake. This contrary behaviour of EDTA and citric acid towards lead genotoxicity suggests the lack of a simple and linear relationship between genotoxicity and phytoavailability and/or Pb uptake in the presence of organic ligands. Indeed, Pb genotoxicity varies with its form/speciation rather than with its total uptake and hence concentration in the presence of organic ligands.

#### 4.4. Correlation between lead-induced genotoxicity and free $Pb^{2+}$ available in nutritive solution

In the present study, the linear correlation ( $r^2=0.94$ ) between lead-induced genotoxicity and  $Pb^{2+}$  concentration (Fig. 4) in nutrient solution under EDTA clearly demonstrated acute genotoxicity associated with this ionic form of lead. In a previous work Pourrut et al. (2008) demonstrated that lead-induced oxidative stress was antagonised by the calcium entry blocker  $LaCl_3$  or high concentrations of  $Ca^{2+}$ , suggesting an important role for the free ion  $Pb^{2+}$ . Sauvé et al. (1998) also found that the toxicological impact of  $Cu^{2+}$  and  $Pb^{2+}$  upon a variety of crop plants, soil organisms and soil microbial processes can be explained to a greater degree by free metal ion activity than the total soil metal concentration.

The mechanism behind this linear relationship between  $Pb^{2+}$  and genotoxicity could be associated with lead-induced oxidative stress. Pourrut et al. (2008) demonstrated a linear dose effect of lead in NADPH-oxidase activation and reactive oxygen species (ROS) production, between 1 and  $10 \mu\text{M}$ . This ROS production could in turn increase DNA alterations and particularly DNA breakdowns at the origin of micronucleus (Yang et al., 1999). Another hypothesis is a possible direct interaction between  $Pb^{2+}$  and DNA (Valverde et al., 2001) and/or inhibition of microtubules by interaction of lead with -SH group of nuclear proteins (Eun et al., 2000).

However, this correlation between lead-induced genotoxicity and  $Pb^{2+}$  concentration is not valid in the presence of citric acid. The contrary behaviour of EDTA and citric acid towards lead-induced genotoxicity is due to differences in their complexing constants.

Indeed, EDTA masks the genotoxic effect of Pb by forming stable and non-toxic complexes with free  $Pb^{2+}$  in solution due to the high stability constant ( $\log K=17.88$ ). In contrast, Pb-CA complex most probably dissociates just before or after uptake due to the lower stability constant ( $\log K=5.67$ ), thus, slightly increasing  $Pb^{2+}$  levels in *V. faba* roots and ultimately genotoxicity. Based on these results it is proposed the lead-induced genotoxicity is directly associated with the uptake of free  $Pb^{2+}$  ions.

Under the application of Pb-EDTA-99, where 99% of the  $Pb^{2+}$  ions were chelated by EDTA, genotoxicity decreased significantly in comparison with Pb alone, but still it was higher with respect to the negative control (NC, Fig. 2B). This was surprising as Pb is assumed to be taken up in the form of Pb-EDTA and Pb induced genotoxicity is activated by free metal ions ( $Pb^{2+}$ ) only. This implies that the presence of 99% chelated Pb should not cause micronucleus induction and the values should be close to those of the NC. Another hypothesis could be a different behaviour of lead at a more dilute scale. In a previous study Sarret et al. (2001) reported that in the case of Pb-EDTA for *Phaseolus vulgaris*, dissociation after uptake is not possible due to strong chelation; however, local rhizosphere acidification could possibly result in the dissociation of the Pb-EDTA complex near the absorption sites of the roots. This production of  $Pb^{2+}$  ions might be responsible for increased micronucleus frequency under our conditions in the presence of Pb-EDTA-99. Moreover, Pb is toxic even at very low concentrations, i.e. 0.5  $\mu M$  (Kopittke et al., 2007) and *V. faba* is highly sensitive to Pb, therefore, micronucleus production by the remaining 1% of free  $Pb^{2+}$  in the Pb-EDTA-99 condition also cannot be ignored.

## 5. Conclusions

The present study is based on the hypothesis that organic ligands could modify both metal uptake and phytotoxicity by changing free metal ions concentration through speciation. The results showed that uptake of Pb by *V. faba* roots varied according to the type of chelate used. EDTA is capable of dose-dependently increasing Pb uptake by *V. faba* roots but citric acid was unable to enhance Pb accumulation by *V. faba* roots following 6 h of Pb exposure. The *V. faba* micronucleus test demonstrates that lead speciation plays a significant role towards its genotoxicity. The results suggest that lead genotoxicity is directly correlated with its free ionic form. EDTA could alleviate lead-induced genotoxicity in *V. faba* roots by forming soluble, stable and non-toxic complexes with lead ions that have a high toxic potential when free. These results also underline that citric acid has no influence on lead-induced genotoxicity to *V. faba* roots due to weak complexation with Pb, which possibly undergoes dissociation producing  $Pb^{2+}$  before or after cell uptake. Finally, our results demonstrate that metal speciation knowledge could improve the interpretation of *V. faba* genotoxicity test performed to test soil quality. Further work could be performed to validate the relationship between free lead cations and genotoxicity and highlight the mechanisms at the cellular scale.

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